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(54) Title: RECONSTITUTED HDL PARTICLES AND USES THEREOF (57) Abstract A reconstituted high density lipoprotein containing particle is disclosed. The particle is useful in removing excess lipid soluble material from a subject to which it is administered, as well as in delivery of lipid soluble pharmaceuticals to subjects or patients.		

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RECONSTITUTED HDL PARTICLES AND USES THEREOF

FIELD OF THE INVENTION

This invention relates to high density lipoprotein (HDL) containing reconstituted particles, and use of these in removing lipid soluble materials from cells, body fluids, and the like. Of particular interest are reconstituted or synthetic HDL particles which are useful in treatment of hypercholesterolemia.

BACKGROUND AND PRIOR ART

10 Normal serum contains a number of lipoprotein particles which are characterized according to their density, namely, chylomicrons, VLDL, LDL and HDL. They are composed of free and esterified cholesterol, triglycerides, phospholipids, several other minor lipid
15 components, and protein. Low density lipoprotein (LDL) transports lipid soluble materials to the cells in the body, while high density lipoprotein (HDL) transports these materials to the liver for elimination. Normally, these lipoproteins are in balance, ensuring proper
20 delivery and removal of lipid soluble materials. Abnormally low HDL can cause a number of diseased states as well as constitute a secondary complication in others.

Under normal conditions, a natural HDL particle is a solid with its surface covered by a phospholipid bilayer that encloses a hydrophobic core. Apolipoprotein A-I and
25 A-II attach to the surface by interaction of the hydrophobic face of their alpha helical domains. In its nascent or newly secreted form the particle is

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disk-shaped and accepts free cholesterol into its bilayer. Cholesterol is esterified by the action of lecithin:cholesterol acyl-transferase (LCAT) and is moved into the center of the disk. The movement of cholesterol ester to the center is the result of space limitations within the bilayer. The HDL particle "inflates" to a spheroidal particle as more and more cholesterol is esterified and moved to the center. Cholesterol ester and other water insoluble lipids which collect in the "inflated core" of the HDL are then cleared by the liver.

Jonas, et al., Meth. Enzym. 128A: 553-582 (1986) have produced a wide variety of reconstituted particles resembling HDL. The technique involves the isolation and delipidation of HDL by standard methods (Hatch, et al., Adv. Lip. Res. 6: 1-68 (1968); Scanu, et al., Anal. Biochem. 44: 576-588 (1971) to obtain apo-HDL proteins. The apoproteins are fractionated and reconstituted with phospholipid and with or without cholesterol using detergent dialysis.

Matz, et al., J. Biol. Chem 257(8): 4535-4540 (1982) describe a micelle of phosphatidylcholine, with apolipoprotein A1. Various ratios of the two components are described, and it is suggested that the described method can be used to make other miscelles. It is suggested as well to use the micelles as an enzyme substrate, or as a model for the HDL molecule. This paper does not, however discuss application of the micelles to cholesterol removal, nor does it give any suggestions as to diagnostic or therapeutic use.

Williams, et al., Biochem & Biophys. Acta 875: 183-194 (1986) teach phospholipid liposomes introduced to plasma which pick up apoproteins and cholesterol. Liposomes are disclosed, which pick up apoprotein in vivo,

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as well as cholesterol, and it is suggested that the uptake of cholesterol is enhanced in phospholipid liposomes which have interacted with, and picked up apoproteins.

5 Williams, et al., Persp. Biol. & Med. 27(3): 417-431 (1984) discuss lecithin liposomes as removing cholesterol. The paper summarizes earlier work showing that liposomes which contain apoproteins remove cholesterol from cells in vitro more effectively than liposomes which do not contain
10 it. They do not discuss in vivo use of apoprotein containing liposomes or micelles, and counsel caution in any in vivo work with liposomes.

It is important to note that there is a clear and significant difference between the particles of the
15 present invention, and the liposomes and micelles described in the prior art. The latter involve a bilayer structure of lipid containing molecules, surrounding an internal space. The construction of liposomes and micelles precludes filling the internal space, however,
20 and any molecular uptake is limited to the space defined between the two lipid layers. As a result, there is much less volume available for pick up and discharge of materials such as cholesterol and other lipid soluble materials than there is for the particles of this
25 invention, which expand in a fashion similar to a balloon, with interior space filling with the material of choice.

The present invention involves the production of reconstituted particles with and without free cholesterol in the bilayer using detergent dialysis. These particles
30 appear when viewed by negative staining transmission electron microscopy (TEM) as discoidal and are transformed to spheroidal particles upon exposure to LCAT. They retain their ability to act as substrates for LCAT and are transformed to spheroidal particles in a fashion similar

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to natural HDL. When reconstituted particles containing 10 percent mole fraction of tritiated cholesterol in the bilayer are exposed to LCAT for 12 hours, almost complete conversion of free cholesterol to cholesterol ester is achieved as determined by thin layer chromatography (TLC).

In summary, synthetic HDL particles are structural and functional analogs of natural HDL particles in that they (1) resemble nascent HDL particles when visualized under negative staining TEM, (2) are substrates for LCAT and (3) function as cholesterol acceptors which deplete cholesterol and other lipid soluble toxins from cells, such as human cells.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example I

LDLs and HDLs were isolated via a series of ultracentrifugation steps. Procedures set out in, e.g., Scanu, et al. Anal. Biochem 44: 576-588 (1971); and Osborne in Segrest, et al. (eds) Meth. Enzym. 128(A); 213-222 (1986) were followed.

20 mls (52 grams) of plasma was used per centrifuge tube. These samples were then spun at 4.0×10^4 RPM for 20 hours at a plasma density of 1.006. This first centrifugation resulted in separation of floated chylomicrons and VLDLs (top 3-4 mls) of each tube. This material was discarded.

The remaining plasma was then pooled into a graduated cylinder, and its density raised to 1.063 by addition of 0.0768 g NaBr/ml of plasma, with complete dissolving of the NaBr. 20 mls (54g) of the dense plasma were distributed per centrifuge tube, and this was centrifuged at 4.0×10^4 RPM for 20 hours. LDLs were present in the

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top 3-4 mls of each sample, and this was removed and dialyzed against TRIS-EDTA buffer (pH 8.0) as described by Hatch, et al., Adv. Lip. Res. 6: 1-68 (1968) overnight at below room temperatures (3 x 1 liter).

- 5 Once the LDLs were removed, the plasma was again pooled and its density raised to 1.2. by adding 0.213g of NaBr/ml of plasma. Again, 20 mls (56g) were placed in tubes, and centrifuged at 4.0×10^4 RPM for 40 hours. Floated HDLs were present in the top 3-4 mls of each tube.
- 10 The HDLs were dialyzed in the same manner as the LDLs, with the clear middle zone reserved for LCAT isolation and purification.

- 15 Once this step was performed, the HDLs were solvent extracted. In this procedure, the HDL-containing solutions were dialyzed against ammonium carbonate buffer (0.01, i.e., 1.572g/ml), overnight at 10°C, followed by shell-freezing (10 mg HDL samples), followed by lyophilization.

- 20 In a second step, the lyophilized HDL was extracted using ethyl alcohol:ether in a 3:1 (V/V) ratio. The EtOH:ether mixture was chilled to 0°C, and 50 mls were used per 10 mg HDL. These were mixed and stored at 0°C for 30 minutes, followed by centrifugation (9×10^3 RPM) for 10 minutes, at 0°C. Supernatant was discarded, and
- 25 this second step was repeated two more times.

- 30 The samples remaining were then extracted in diethyl ether for 30 minutes, and centrifuged as described supra. This step is repeated two more times, and the final supernatant was aspirated off and dried under a stream of nitrogen.

The apoprotein or apo HDL which was obtained was then tested for residual phospholipids and cholesterol.

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Example II: Making Reconstituted HDL Particles

A. Rehydration of Apo-HDL

7.2 mg of Apoprotein containing HDL (5.02 mg are Apoprotein AI and Apoprotein AII) were dissolved in 1 ml
5 TRIS-EDTA containing 3M guanidine HCl (0.287 g/ml) at pH 8.0 in a 12 x 75 mm test tube capped with paraffin. This was placed in a rotating shaker and allowed to dissolve for a minimum of one hour. If necessary, sonication was used to disperse the protein.

10 The resulting solution was dialyzed against 2 liters of TRIS-EDTA buffer (10 mM NaCl, 10 mM EDTA, 1mM azide; pH 8.0), at below room temperature. The tubing used in the dialysis has a MW cutoff of 12,000. This is because Apo AI and AII constitute 70% of Apo HDL, and 30% more protein
15 was used to take into consideration the loss of proteins with molecular weight less than 12,000. A dialysis tube which will not lose proteins of this size is therefore necessary.

B. Preparing lipid:cholate and protein:cholate dispersion.

20 To make particles without cholesterol, 14.2 mg of egg phosphatidylcholine (EPC) was weighed out into a tared, 13 x 100 mm test tube, with addition of 1 ml chloroform to dissolve it completely. This was dried to a thin film
25 under N₂.

To make particles with cholesterol, 12.74 mg of EPC was weighed and dissolved as described supra, and 69.6 ul of a 10 mg/ml stock solution of cholesterol in chloroform was added. Drying was as above.

30 Whether cholesterol containing or cholesterol free films were prepared, 3 mls of 18mM sodium cholate was added to the film containing tubes. The tubes were sealed

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and sonicated for about 1 minute until all of the lipid was dispersed.

The apoprotein solution prepared as described supra was transferred to a 15 ml Falcon tube, and spun to pellet undissolved material. The apoprotein solution was transferred to a disposable test tube (13 x 100 mm; 1 ml of solution), and 1 ml of 36 mM sodium cholate was added. The final concentration of cholate in this apoprotein:cholate solution was 18 mM.

10 C. Preparing Reconstituted HDL Particles

The lipid cholate solution was added to the protein cholate solution, and the tube containing these was sealed, swirled gently, and incubated overnight at 10°C. The sample was then dialyzed against 3, 2 liter changes of TRIS-EDTA, at 10°C. It is important that the sample remain at 10°C through the entire dialysis procedure. As described supra, the same conditions are in force regarding dialysis tubing.

This procedure produced reconstituted particles which were transferred to a 15 ml Falcon tube and centrifuged to remove large materials. Reconstituted HDL particles remained in the supernatant and could be filter sterilized, e.g., using a 0.22 μ m syringe filter.

25 Example III: The Use of Synthetic HDL to remove Cholesterol from Cells

The ability of cholesterol-free synthetic HDL particles to remove cholesterol from cholesterol-loaded human peripheral blood monocyte-derived macrophages was determined. Macrophages were grown in delipidated heat-treated human serum (DHS) for two days to up-regulate the LDL receptors. The cells were fed LDL and acetylated

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LDL for two days to load the cells with cholesterol. The LDL was removed and the cells were rinsed with DHS. The cells were incubated in DHS containing approximately 10 mg/ml LCAT and 0.1 mg/ml (protein) reconstituted HDL particles for four hours. After loading, cells appeared to contain large oil droplets and after a four hour incubation period, they appeared to be almost completely depleted of these droplets. Two replicated experiments were conducted. The synthetic HDL particles acquired 46 and 62 ug/ml cholesterol, respectively.

Example IV: Demonstration that Injected Synthetic HDL Particles can Increase Plasma HDL Concentrations In Vivo

The effect of injected synthetic HDL particles on plasma HDL concentration in the heterozygous Watanabe Heritable Hyperlipidemic Rabbit (WHHL) was determined. Rabbit HDL was purified from normal rabbit plasma and used directly (natural HDL) or used to produce HDL apoproteins which in turn were combined with egg phospholipid to make synthetic HDL as described above under "Methods". Three male 3 kg heterozygous WHHL rabbits were randomly assigned to one of the following experimental treatments: (1) a saline control, (2) a synthetic HDL or (3) a natural HDL treatment. The saline control animal received a single injection of 20 ml of sterile normal saline. The synthetic HDL and natural HDL treatment animals received 100 mg of synthetic or natural HDL (measured as phospholipid) injected in 20 ml of saline. HDL phospholipid was measured 30 to 60 minutes after injection. Table 1 shows the observed changes in HDL phospholipid assuming that, after injection the synthetic HDL behaves as natural HDL.

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Table 1. Observed changes from baseline in HDL phospholipid (mg/dl) after injection of saline, natural or synthetic HDL.

	<u>Saline</u>	<u>Natural HDL</u>	<u>Synthetic HDL</u>
5 Baseline	78.0	78.0	53.2
Post 30 Min	---	100.4	78.0
Post 60 Min	83.6	97.6	72.8

Example V: Demonstration that Injected Synthetic HDL is Accepted as Natural HDL in Animals

10 This study demonstrated that injected synthetic HDL particles are accepted as natural HDL in animals. Rabbit HDL was purified from normal rabbit plasma and used directly (natural HDL) or used to produce HDL apoproteins which in turn were combined with egg phospholipid to make
15 synthetic HDL as described above under "Methods". Three male 3 kg heterozygous WHHL rabbits were given an injection of saline, synthetic or natural HDL. The saline control animal received an injection of 20 ml of sterile normal saline. The synthetic and natural HDL treatment
20 animals received 100 mg of synthetic or natural HDL (measured as phospholipid) injected in 20 ml of saline. Blood was drawn from each animal at 30 and 60 minutes after injection. A 1 ml aliquot of whole plasma from each sample was passed down a rabbit anti-LDL sepharose column
25 (sheep anti-rabbit LDL covalently linked to Sepharose

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CL-4B) to remove beta lipoproteins. The plasma eluting from the columns was essentially all HDL. The columns were washed with three additional mls of saline. HDL cholesterol was measured by the standard enzymatic
5 cholesterol assay.

Table 2 shows the observed changes in HDL cholesterol assuming that after injection the synthetic HDL behaves as natural HDL. HDL cholesterol quadrupled in the animal that received natural HDL and almost doubled in the animal
10 receiving synthetic HDL. The difference in response between natural versus synthetic particles is due to the fact that the natural HDL injection contained nature cholesterol-filled particles, whereas the synthetic particles were completely devoid of cholesterol and were
15 in the process of filling when the measurements were taken. The assay used specifically measures the cholesterol content.

20 Table 2. Observed changes from baseline in HDL cholesterol (mg/dl) after injection of saline, natural or synthetic HDL.

	<u>Saline</u>	<u>Natural HDL</u>	<u>Synthetic HDL</u>
Baseline	24.9	10.3	12.4
Post 30 Min	23.0	43.7	22.9
Post 60 Min	23.7	43.7	22.9

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Example VI: Demonstration that Injected Synthetic HDL is
Converts Natural HDLc to HDL2 in Animals

This study demonstrated that synthetic HDL converts mature cholesterol-rich HDL to a smaller particle that can accept additional cholesterol and resembles HDL2 in animals. Rabbit HDL was purified from normal rabbit plasma and used directly (natural HDL) or used to produce HDL apoproteins which in turn were combined with egg phospholipid to make synthetic HDL as described above under "Methods." Two male 3 kg heterozygous WHHL rabbits were given an injection of either synthetic or natural HDL. The synthetic and natural HDL treatments received 100 mg of I^{125} labelled synthetic or unlabeled natural HDL (measured as phospholipid) injected in 20 ml of saline. Blood was drawn from each animal at 30 and 60 minutes after injection. Whole plasma from the 30 and 60 minute samples from each animal were pooled, the natural HDL sample was spiked with I^{125} -labeled synthetic HDL particles and 3 mls of each sample were run on 25 x 100 cm agarose A 0.5M columns. In addition, a sample of synthetic HDL particles was spiked with a small volume of I^{125} -labeled synthetic particles and run on a column. Figure 1 shows the elution profile of counts per minute as a function of elution volume in mls. The majority of HDL in plasma occurs as a large cholesterol-rich particle known as HDLc which elutes in approximately 380 mls. The synthetic HDL is a smaller particle which behaves like human HDL2, eluting in approximately 400 mls. HDL2 is generally considered to be the anti-atherogenic HDL. This demonstrates the conversion of the plasma in the animal from the mature cholesterol rich form of HDLc to a form which can accept additional cholesterol and which resembles HDL2.

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The synthetic HDL particles described herein will be seen by those skilled in the art to be useful in various biological and biochemical application. It has long been known that liposomes, e.g., are useful in drug delivery systems. (See, e.g., Fountain, et al., U.S. Patent No. 4,610,868; Ryan, et al., U.S. Patent No. 4,544,545; Ash, et al., U.S. Patent No. 4,448,765; and Kelly, U.S. Patent No. 4,551,288, as some examples of the literature on this point). As has been pointed out supra, however, liposomes have extremely limited capacity for carrying any substance. The reconstituted particles of this invention, due to their carrying capacity, represent a substantial improvement over liposomes and micelles as used in drug delivery.

Additionally, the particles described therein will be seen to be useful in removing lipid soluble wastes including, but not limited to, cholesterol, cholesterol esters, insecticides such as DDT, and carcinogens such as dioxin. In each case, the particles supplement HDL levels in a subject to which they are administered, and act to transport organic or inorganic lipophylic substances to the liver, which is the site of lipid soluble waste excretion.

Typical diseases which can be treated by administration of these particles include, but are not limited to, coronary atherosclerosis, and hyperlipidemia resulting from, e.g., diabetes and nephrotic diseases.

In each case, treatment will vary because each subject will differ and require different amounts of particles. As HDLs occur normally in biological systems and are processes rather quickly, they are non-toxic and problems associated with, e.g., "LD" determinations for new pharmaceuticals are not an issue. Intravenous administration is the accepted mode of delivery.

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Another application of the invention is in separation of harmful substances from body fluids which contain excess or harmful lipids. In this application, synthetic HDL particles are linked to an inert carrier such as, but not limited to, sepharose columns or other stationary supports. The linkage is accomplished by any of the known materials which are used to attach substances to carriers, such as cyanogen bromide. Via appropriate catheters, tubing, etc., body fluids are removed and passed over the inert column with attached synthetic particles, for a time sufficient for the HDL particles to pick up and remove the lipid materials. In this way, toxic lipid soluble substances such as dioxin, or lipid materials which are present in fluids, such as excess blood cholesterol, are removed. In the case of cholesterol, synthetic HDL particle containing columns are used "in line" with LDL-pheresis columns used in connection therewith. In this situation the HDL column cleanses naturally occurring HDL of cholesterol and returns the ability of natural HDL as a cholesterol carrier thereto.

The HDL particles function, as described supra, as delivery vehicles for drugs. One key example is the delivery of pharmaceutically active phospholipids, or other drugs which can be attached to the lipid or protein portion of the particle either before, during, or after particle formation. One example is a particle in which is incorporated a bifunctional reagent which inserts one end into the polar head portion of the particle phospholipid, with a site for drug attachment at the other functional moiety.

Additionally, the synthetic HDL particles described herein will be seen by those skilled in the art to be effective in various diagnostic procedures. As synthetic HDL particles can have a hydrophobic core, various

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determinable substances, or "tags", such as chromogenic, chromophoric, fluorogenic and other labelled reagents can be incorporated therein in order to measure activity of lipid active factors in the plasma of a patient. HDL particles constructed without hydrophobic cores accept lipid in the manner described supra, from subject plasma, and the lipid donating potential of the subject's plasma quantitatively measured as the amount of chromogen accepting capacity of synthetic HDL particles following "inflation" or "filling" with plasma lipid. Examples, but not limits, on the lipid active factors measurable thereby are lecithin cholesterol acetyltransferase (LCAT); cholesterol ester transfer protein (CET); cellular HDL receptor activity; plasma cholesterol transport potential; and removal of biotoxins. The last of these is performed in vitro such as is described supra. Each of these examples involve mechanisms which the art views as involved in cholesterol ester (nonpolar lipid) transport, and atherosclerosis. In situations where chromogens are used, these are screened for their ability to partition into the hydrophobic core of inflated synthetic particles after reaction with LCAT in the presence of a cholesterol donor. Acceptable chromogens include those which can be spectrophotometrically measured at from 300 to 500 nm.

While there have been described what are at present considered to be preferred embodiments of this invention, it will be obvious to one skilled in the art that various changes and modifications may be made therein without departing from the invention, and it is, therefore, aimed to cover all such changes and modifications as fall within the true spirit and scope of the invention.

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WE CLAIM:

- 1 1. A reconstituted particle comprising at least one high
2 density lipoprotein and at least one lipid molecule.
- 1 2. Particle of claim 1, wherein said high density
2 lipoprotein contains apoprotein I.
- 1 3. Particle of claim 1, wherein said high density
2 lipoprotein contains apoprotein II.
- 1 4. Particle of claim 1, wherein said high density
2 lipoprotein contains apoprotein I and apoprotein II.
- 1 5. Particle of claim 1, wherein said lipid molecule is a
2 phospholipid.
- 1 6. Particle of claim 5, wherein said phospholipid is
2 phosphatidylcholine.
- 1 7. Particle of claim 1, wherein said particle further
2 comprises cholesterol.
- 1 8. Method of lowering the amount of a lipid soluble
2 substance in a subject hyperlipidemic for said
3 substance comprising administering to said subject a
4 lipid lowering amount of a pharmaceutically
5 acceptable reconstituted particle containing a high
6 density lipoprotein and a lipid in which said
7 substance is soluble.
- 1 9. Method of claim 8, wherein said lipid soluble
2 substance is cholesterol.

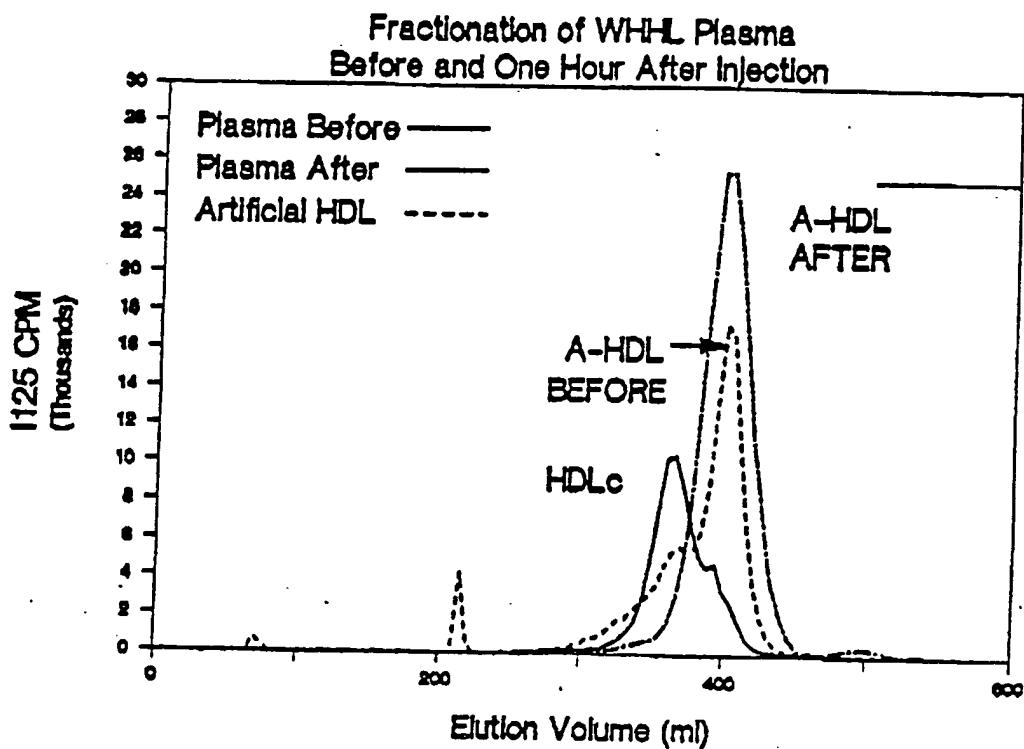
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- 1 10. Method of claim 8, wherein said lipid soluble
2 substance is an insecticide.
- 1 11. Method of claim 10, wherein said insecticide is DDT.
- 1 12. Method of claim 8, wherein said lipid soluble
2 molecule is a carcinogen.
- 1 13. Method of claim 12, wherein said carcinogen is
2 dioxin.
- 1 14. Method of claim 8, wherein said substance is an
2 endotoxin.
- 1 15. Method for enhancing drug delivery in a subject
2 comprising incorporating a drug to be delivered in a
3 reconstituted particle containing a high density
4 lipoprotein and a lipid and administering said
5 reconstituted particle containing said drug to said
6 subject in an amount sufficient to cause a desired
7 effect thereto.
- 1 16. Method of diagnosing lipid donating potential of a
2 subject's plasma comprising administering to said
3 subject a reconstituted HDL particle containing a
4 determinable substance for a time sufficient for said
5 particles to accept said lipids from said plasma and
6 determining the chromogen in said particles after
7 donation of said lipids, wherein measurement of said
8 chromogen determines lipid donating potential of said
9 plasma.

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- 1 17. Method of claim 16, wherein said method comprises
- 2 measuring lecithin cholesterol acetyltransferase,
- 3 cholesterol ester transfer protein, cellular HDL
- 4 receptor activity, plasma cholesterol transport
- 5 potential, or biotoxin removal.

Figure 1. Fractionation of rabbit plasma one hour after injection with synthetic HDL or natural HDL. Synthetic HDL particles prior to injection are also shown.



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/01725

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3 According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): C07K-15/16; A61K-37/02; A61K-37/22, US: 514/2; 514/21; 530/359		
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched *</div> <div style="display: flex; justify-content: space-between;"> <div style="width: 45%; border-bottom: 1px solid black;">Classification System 1</div> <div style="width: 45%; border-bottom: 1px solid black;">Classification Symbols</div> </div> <div style="margin-top: 10px;"> U.S. 530/359; 514/2; 514/21 </div>		
<div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *</div> Computer database searches on A.P.S., and on Biosis/CAS-online.		
III. DOCUMENTS CONSIDERED TO BE RELEVANT 14		
Category *	Citation of Document, 15 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 16
X - Y	Segrest et al, "Methods in Enzymology", Vol.128, part A, published 1986, by Academic Press (Orlando, Florida, USA), see pages 3 to 41, 223 to 246, and 554 to 582, especially pages 554 to 569.	1-7 ----- 1-9
X - Y	Nature, Vol.265, issued 13 January 1977 (London, UK), Tall et al, "Solubilization of phospholipid membranes by human plasma high density lipoproteins", pages 163 to 164.	1-7 ----- 1-9
X - Y	Clinica Chimica Acta, Vol.131.No.3, issued 1983 (The Netherlands), Malmendier et al, "In vivo metabolism of human apoprotein A-I-phospholipid complexes. Comparison with human high density lipoprotein-apoprotein A-I metabolism.", pages 200 to 211.	1-7 ----- 1-9
X - Y	The Journal of Biological Chemistry, Vol.257, No.8, issued 25 April 1982 (Rockville, MD, USA), Matz et al, "Micellar Complexes of Human Apolipoprotein A-I with Phosphatidylcholines and Cholesterol Prepared from Cholate-Lipid Dispersions", pages 4535 to 4540.	1-7 ----- 1-9
<div style="display: flex; justify-content: space-between; font-size: small;"> <div style="width: 45%;"> * Special categories of cited documents: 15 "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </div> <div style="width: 45%;"> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "d" document member of the same patent family </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 2 <div style="border: 1px solid black; padding: 2px; display: inline-block;">15 August 1988</div>		Date of Mailing of this International Search Report 2 <div style="border: 1px solid black; padding: 2px; display: inline-block; font-size: 1.2em;">0 8 SEP 1988</div>
International Searching Authority 1 <div style="border: 1px solid black; padding: 2px; display: inline-block;">ISA/US</div>		Signature of Authorized Officer 19 <div style="border: 1px solid black; padding: 2px; display: inline-block;"> P. Kushan </div>

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Biochimica et Biophysica Acta, Vol.875, No.2,	1-7
-	issued January 1986 (Amsterdam, The Netherlands),	_____
Y	Williams et al, "Uptake of endogenous cholesterol by a synthetic lipoprotein", pages 183 to 194.	1-9
X	Biochimica et Biophysica Acta, Vol.513, No.2,	1-7
-	issued November 1978 (Amsterdam, The Netherlands),	_____
Y	Tall et al, "Interaction of cholesterol, phospholipid and apoprotein in high density lipoprotein recombinants", pages 185 to 197.	1-9
Y	US, B, 4,499,184 (GOODHUE), 12 February 1985, see entire document.	14

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter ¹³ not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages *	Relevant to Claim No. *
X	FR, A, 2,559,669 (NABAVI), 23 August 1985, see	1,5-9
-	abstract and page 1 especially.	
Y		1-9
Y	US, B, 4,544,630 (ZIEGENHORN ET AL), 01 October 1985, see abstract, columns 1 to 5 especially.	16-17
Y	US, B, 4,522,803 (LENK ET AL), 11 June 1985, see columns 3 and 4 especially.	15